



The Effect of Superparamagnetic Fe₃O₄ Nanoparticles Combined with Quercetin on Breast Cancer Cell Line (MCF-7)

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ABSTRACT

Background: Magnetic nanoparticles attract increasing interest due to their use in cancer therapy and as drug carriers for several other diseases. The present study investigates the physicochemical properties of quercetin-conjugated superparamagnetic Fe₃O₄ nanoparticles and their effects on breast cancer cell line MCF-7. **Methods:** A simple precipitation method was used to prepare the Poly Ethylene Imine (PEI)-coated Fe₃O₄ nanoparticles; they were then conjugated with flavonoid-compound quercetin on the surface via carboxylic/amine group using nanoprecipitation method. Then, the physical and chemical parameters were calculated using Zeta-sizer, scanning electron microscopy (SEM), and extract release patterns at 37 and 42 °C. Finally, the toxicity level of this quercetin-conjugated nanosystem on the MCF-7 cells was investigated by MTT assay. **Results:** The results showed that the prepared nanosystem attained about 74% of quercetin inclusion, 91.2 nm size, 65.1 mV zeta potential, spherical morphology and a controlled release. Compared to Fe₃O₄ nanoparticles and pure quercetin, MTT and microscopy analysis revealed that quercetin-conjugated Fe₃O₄ nanoparticles induced considerable cytotoxicity, and morphology changes against MCF7 cells. **Conclusion:** Quercetin-conjugated Fe₃O₄ nanoparticles have appropriate physicochemical properties; they can be a suitable carrier for drug delivery and a promising therapy for candidates.

Keywords: Nanoparticles; Quercetin; Fe₃O₄; Breast cancer

Introduction

In the treatment of cancer, depending on the type, the degree of cancer progression, the extent of the disease, and the patient's condition, a combination of different methods such as surgery, radiotherapy, and chemotherapy are used. Despite the creation of many side effects, these therapeutic

methods for advanced cancers usually do not lead to positive results (Ramar *et al.*, 2015).

Despite the progression in using synthetic drugs or antitumor herbal compounds, serious challenges, including low solubility, low stability, low absorption, and oxidation of some active

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substances remain unsolved (Bhalla *et al.*, 2013). Nanotechnology has been able to reduce or eliminate many problems by preparing medicinal nanocarriers including liposome, neosome, and superparamagnetic nanoparticles (Engineer *et al.*, 2011, Haghirsadat *et al.*, 2016).

Well-designed superparamagnetic nanoparticles with ultra-small size, monodisperse shape, colloidal stability, modified surface area, magnetic enhancement, and cellular uptake appear to be suitable carriers/agents for biomedical applications (Badruddoza *et al.*, 2012, Kakran *et al.*, 2011). Some researchers have already discovered that the commercial anticancer drugs are loaded on the magnetite for successful function of chemotherapeutic agents (Engineer *et al.*, 2011). The surface of nanoparticles related to the superparamagnetic Fe₃O₄ is modified with, poly ethyl imine (PEI), peptides, polyvinyl alcohol (PVA), proteins, carbohydrates, etc. This process helps loading the drug and controls the release (Reddy *et al.*, 2012, Revia and Zhang, 2016). The quercetin encapsulated on the nanocarriers of PEI coated Fe₃O₄ is among the possible techniques to solve the above problems. It also increases the drug's bioavailability (Lopes *et al.*, 2020). Quercetin is a herbal flavonoid compound with anti-proliferative properties that synergistically increases the anti-tumor effect of chemotherapy drugs (Kumari *et al.*, 2010, Wang *et al.*, 2016).

This fact encourages the researchers to synthesize the nanoparticles related to quercetin-conjugated magnetite and evaluate their in-vitro anticancer activity. Synthesis method influences monodispersed size and shape of the magnetic nanoparticles.

Moreover, there are limitations regarding agglomeration, non-uniform distribution of particle, and instability (Ó'Connor, 2012). The aim of the current research is to prepare magnetic Fe₃O₄ nanoparticles conjugated to quercetin and study their physicochemical characteristics. For this purpose, the cytotoxicity effect of prepared nanoparticles on MCF7 cell line is determined and confirmed.

Materials and Methods

This study was done in a laboratory using cell line breast cancer (MCF-7). It was obtained from Pasteur Institute cell bank (Tehran, Iran). For medicinal treatment of the cells, the drug quercetin purchased from Sigma was used. Quercetin stock solution was prepared by dissolving it in DMSO (Dimethyl sulfoxide, 0.1% sterile).

Cell lines and culture conditions: Human breast cancer cell line MCF-7 (obtained from Pasteur Institute, Tehran, Iran), was cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with glutamine and 10% fetal bovine serum (FBS) in a humidified atmosphere (37 °C, 5% CO₂, 95% air).

Quercetin standard diagram in water and phosphate buffered saline (PBS): In order to draw drug calibration graph and obtain its line equation, different concentrations of quercetin drug stock were made in water and PBS using the standard series method. Then, their optical absorption was measured by a spectrophotometer at the maximum wavelength of quercetin, 375 nm, with 3 repetitions; after that, the calibration curve was drawn (Majdzadeh *et al.*, 2018).

Synthesis of magnetite (Fe₃O₄) nanoparticles: The synthesis of magnetic nanoparticles was done using chemical co-precipitation method (Yang *et al.*, 2020, Yazdani and Seddigh, 2016). For this purpose, 1.25 grams of FeCl₂·4H₂O and 3.4 grams of FeCl₃·6H₂O were dissolved in 100 ml of deionized water by a strong shaker. Then, 5 ml of NaOH (0.3 M) was gradually added while stirring vigorously for 30 to 40 minutes at a temperature of 60 °C. The result was a black colored mixture which indicated the formation of Fe₃O₄ sediment, and the black products were separated with a permanent magnet. The sediment should be washed 3 times with 70 ml of deionized water. To prevent accumulation, 0.02 grams of tetraethylammonium perchlorate should be added to the synthesized nanoparticles. After that, it was dried at 50 °C for 16 hours in a vacuum drying chamber (Hakimian *et al.*, 2023).

Coating the surface of magnetic nanoparticles with poly ethylene imine polymer: After the synthesis of magnetic nanoparticles by co-precipitation method, magnetic nanoparticles and poly ethyl eneimine were incubated in a mass ratio (1:2) at room temperature for 24 hours at pH=9. Magnetic nanoparticles coated with poly ethyl eneimine were washed with distilled water and methyl alcohol at least 3 times and separated by centrifugation; following that, the modified nanoparticles were dried at 60 °C for 24 hours (Zhao *et al.*, 2020).

Conjugation of Fe₃O₄ nanoparticles with quercetin (QCMNP): In order to draw the quercetin calibration graph, different concentrations of the drug (Sigma, USA) and stock solution (dissolved in DMSO) were made in PBS, their optical absorption was measured by a spectrophotometer at the maximum wavelength of 375 nm with 3 repetitions, and then, the calibration curve was drawn (Majdzadeh *et al.*, 2018).

The nanoprecipitation method was used to prepare quercetin-conjugated nanoparticles with small modifications. Generally, quercetin dehydrate is soluble in acetone and insoluble in water. A specified level of quercetin dehydrate was dissolved in 3 ml of acetone. After that, 100 mg of nanoparticles of polyethyleneimine polymer coated magnetite was dissolved in 30 ml of dimethyl sulfoxide (DMSO) and DI water under sonication. With continuous ultrasonication, the quercetin solution was added and stirred for 24 hours at room temperature. By adding KOH solution, the pH level of the solution was determined to be 10. To remove any organic impurity or unbound drug, the yielded black color precipitate was cleansed with distilled water. The resultant drug loaded magnetite nanoparticles were collected using external magnet and dried using lyophilizer/ freeze drier. These drug conjugated magnetite nanoparticles were stored below the room temperature for further studies (Kumar *et al.*, 2014, Sun *et al.*, 2013).

Determination of quercetin-loading and encapsulation's efficiency: To evaluate the

quercetin loading and encapsulation, 5 mg of quercetin-loaded nanoparticles was dissolved in 10 ml of PBS, and after 30 minutes, it was centrifuged at 12,000 rpm. Then, the absorbance at 375 nm was measured by a UV–visible spectrophotometer set. The concentration of the drug in the supernatant was determined, and loading and encapsulation efficiency was calculated as follows:

$$\text{Loading efficiency} = W_0/W \times 100\% \quad (1)$$

$$\text{Encapsulation efficiency} = W_0/W_1 \times 100\% \quad (2)$$

W: the weight of Fe₃O₄ nanoparticles,

W₀: the weight of quercetin loaded on the Fe₃O₄ nanoparticles,

W₁: the initial level of quercetin added into the system (Kumar *et al.*, 2014).

Determination of particle size and zeta potential: The zeta potential of the formulation and the size of nanoparticles obtained from this formulation were measured using a zetasizer device (Malvern Instruments company, model nano-zetasizer), at room temperature and an angle of 90 °C.

Determination of in-vitro quercetin releasing: The releasing activity of quercetin from Fe₃O₄ nanoparticles was investigated in the pH of 7.4 and 5.5. The drug conjugated magnetite nanoparticles were suspended in PBS and transferred to a dialysis bag. The bag was placed in the dialysis tubing (MW3500, MD24, USA) and incubated in 250 ml of PBS (pH 7.4) under shaking. The release study was performed at 37.0±0.5 °C. At pre-determined time intervals, 5 ml of the aqueous solution was withdrawn and replenished with 5 ml of fresh buffer solution. The amount of drug release was estimated by measuring absorbance at 375 nm for quercetin (initial drug concentration in dialysis bag was 40 lg/ml) using UV–Visible spectrophotometer. The amount of drug release and the percentage of quercetin from Fe₃O₄ nanoparticles was measured for various time intervals. The UV–Vis spectrophotometer (Hitachi, Japan) was used for this analysis (Kumar *et al.*, 2014)

Determination of in-vitro-cell uptake of nanoparticles: For evaluating cell uptake of

quercetin, 15×10^4 MCF7 cells were grown in 6-well plates for 24 hours, then the cells were incubated with formulas of free quercetin, quercetin conjugated in iron oxide magnetic nanoparticle (100 nM), and magnetic nanoparticle without drug for 3 hours at 37 °C. DAPI dye was used to stain the nucleus of cells that can be seen in blue color using a fluorescent microscope. On the other hand, quercetin is seen in green color under the microscope due to its fluorescent property.

In-vitro cytotoxicity assay of nanoparticles on MCF7 cells: The MTT assay was used to evaluate the cell viability of treated cells versus controls (Horton *et al.*, 2011). About 8×10^4 cells/well were seeded in 96-well plates and kept at 37 °C, 5% CO₂ for 24 h. Then, different concentrations of free quercetin, quercetin-conjugated Fe₃O₄ nanoparticles (QCMNPs), and magnetic nanoparticles without drug were added separately to the wells and kept at 37 °C in a CO₂ incubator for 24 h. Next, MTT assay was done, and the absorbance of each well at 570 nm was measured by an ELISA plate reader. The percentage of cell viability was calculated based on this formula:

$$\% \text{ Viability} = ([\text{OD}] \text{ test} / [\text{OD}] \text{ control}) \times 100$$

Ethical considerations: This study was approved by the ethics committee affiliated with Yazd Shahid Sadoughi University of Medical Sciences (Ethics code: IR.SSU.MEDICINE.REC.1399.092).

Data analysis: Prism software, t-test, and ANOVA were used to statistically check the results, and the significance of the results was measured by P-value <0.05.

Results

Comparing the standard curve of the quercetin concentrations: Based on the Beer-lambert law, a linear relationship between absorption and concentrations of quercetin in water (**Figure 1**), and phosphate buffer saline (PBS, **Figure 1**) were obtained, and the calibration chart was drawn. The graphs had an acceptable R² (R square) above 98%. Next, the first degree equation was used in the calculation of drug loading and release. The loading rate of quercetin in the nanoparticle was $74.00 \pm 5.74\%$, calculated by referring to the

calibration chart of PBS.

Zeta potential and favorable size of the quercetin-loaded Fe₃O₄ nanoparticles: Data obtained from the DLS device showed that the size of nanoparticles containing quercetin was about 91.2 nm (**Figure 3**), and their surface charge (zeta potential) was about 65.1 mV (**Figure 4**). The SEM-image analysis (**Figure 5**) indicated that the nanosystem containing quercetin has suitable characteristics: a favorable appearance, a homogenous morphology, a uniform-size distribution, and a spherical structure. Also, images revealed no adhesions between the particles. Measurement of the nanoparticle diameter with the microscopy technique reflected the accuracy of the diameter measured with the DLSE device.

Two-phase pattern of the drug release by Fe₃O₄ nanoparticles: The release of quercetin drug from the nanoparticle was carried out using the dialysis method in several time intervals at two temperatures of 37 and 42 °C, which are the normal body and cancer cells temperatures, respectively. The rate of quercetin release from magnetic nanoparticles was 70% in the first 48 hours (**Figure 6**).

Nanoparticle uptake and quercetin entry into the nucleus: To investigate the cellular uptake of nanoparticles and drug entry into the nucleus, DAPI dye that binds to A-T rich regions in DNA was used to stain the nucleus. Therefore, using a fluorescent microscope with DAPI, the nucleus of the cells was seen in blue color; with quercetin, on the other hand, the nucleus was seen in green due to its fluorescent properties (**Figure 7**). Herein, the blue-green color of the nucleus in **Figure 7** represented DAPI plus quercetin merging in the nucleus (**Figure 7**).

In-vitro cytotoxicity of quercetin-conjugated Fe₃O₄ nanoparticles: Cytotoxicity of the pure quercetin and quercetin conjugated Fe₃O₄ nanoparticles on MCF-7 cells was studied. In this way, the free and the encapsulated drugs were applied in specific concentrations on the MCF-7 cells with three repetitions for the purpose of IC₅₀ detection. (the required concentration of the drug for 50% inhibition of cell growth compared to the

control group). Based on the results and the graph obtained (Figures 8,9), the amount of IC50 for free quercetin was 96.44; on the other hand, the amount of IC50 on MCF-7 cells in the same conditions was 49.59. Cytotoxicity results indicated that the IC50 drug encapsulated in nanoparticles was far less than the amount of IC50 of free drug in a certain concentration; however, encapsulating the effective drug showed a greater

effect on the lethality of MCF-7 cells. Moreover, empty Fe3O4 nanoparticles did not show any cytotoxicity on the MCF-7 cell line (figure 10).

Fe3o4 containing quercetin and Fe3o4 without drugs were calculated in 48 hours by checking the significance of cytotoxicity in different concentrations based on the two-way-Anova test and reported based on the statistical criterion of Pvalue.

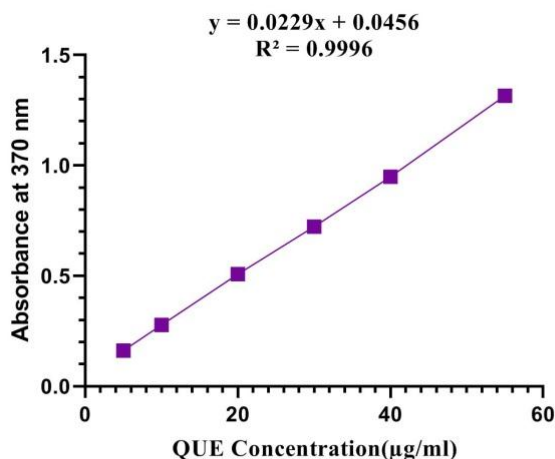


Figure 1. Standard diagram of quercetin in water.

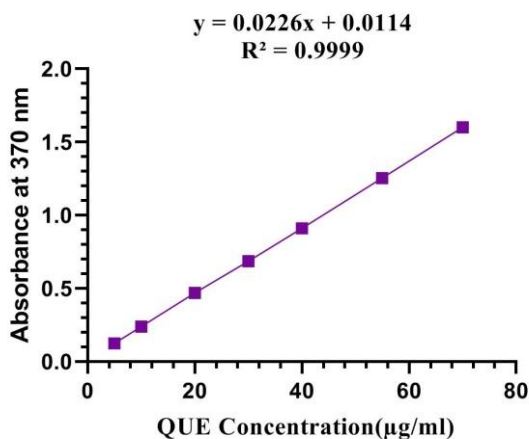


Figure 2. Standard diagram of quercetin in PBS.

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	30.3 nm	1.9 nm	29.9 nm
2	0.00	98.0 nm	9.3 nm	97.2 nm
3	---	--- nm	--- nm	--- nm
Total	1.00	30.5 nm	4.1 nm	29.9 nm

Cumulant Operations

Z-Average : 91.2 nm

PI : 0.342

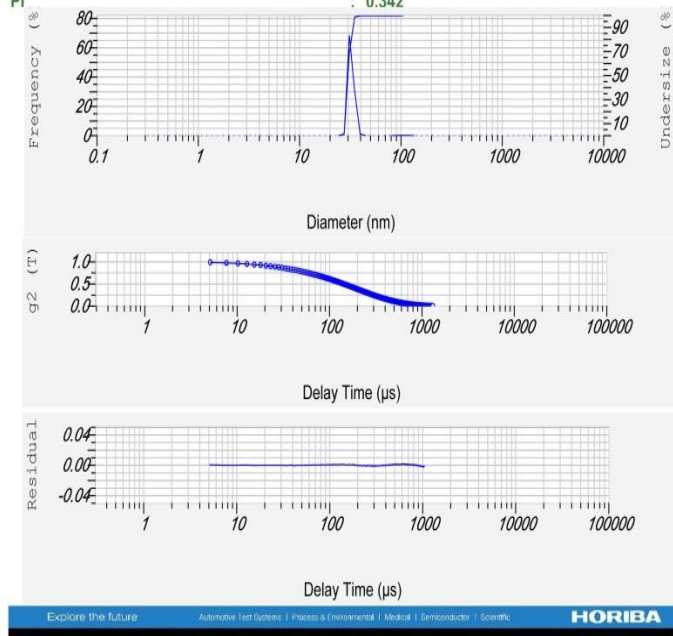


Figure 3. Size of Fe³⁺O₃ nanoparticles containing quercetin drug.

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	65.1 mV	0.000506 cm ² /Vs
2	--- mV	--- cm ² /Vs
3	--- mV	--- cm ² /Vs

Zeta Potential (Mean) : 65.1 mV

Electrophoretic Mobility Mean : 0.000506 cm²/Vs

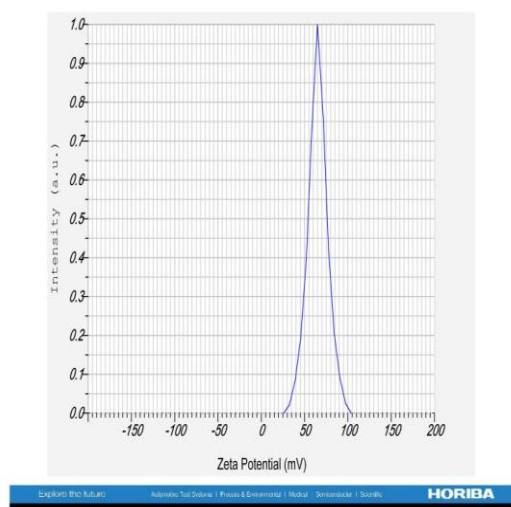


Figure 4. Zeta potential of Fe³⁺O₃ nanoparticles containing quercetin.

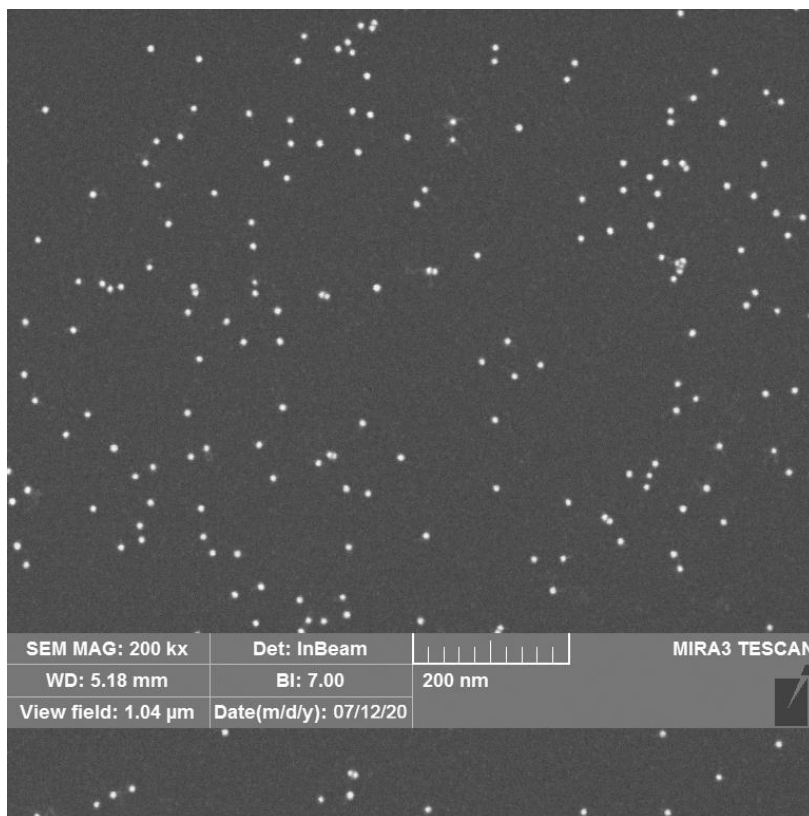


Figure 5. SEM images of the optimal formulation of quercetin-loaded Fe₃O₄ nanoparticles.

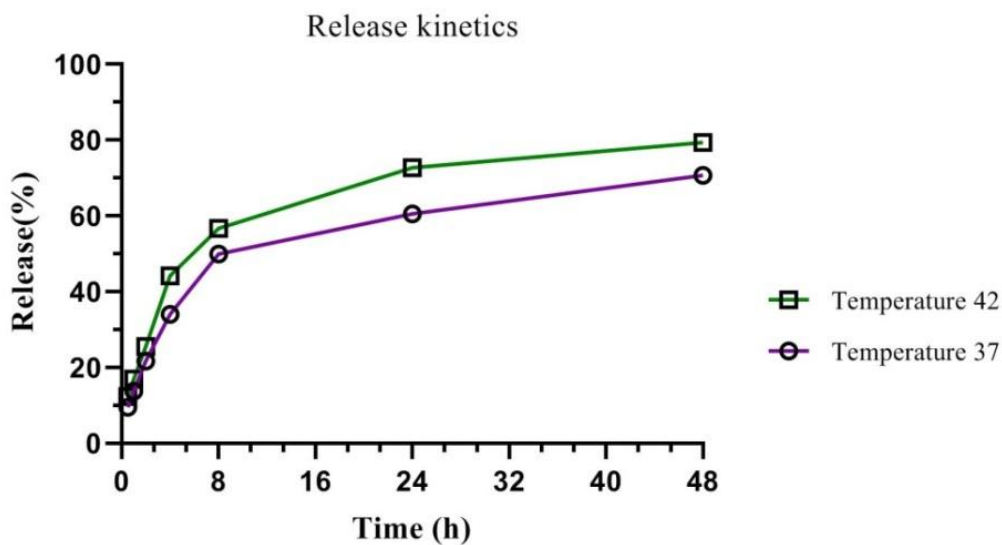


Figure 6. Quercetin drug release pattern at 37 and 42 degrees.



Figure 7. Investigating the cellular entry of the magnetic system containing quercetin into MCF-7 cancer cells.

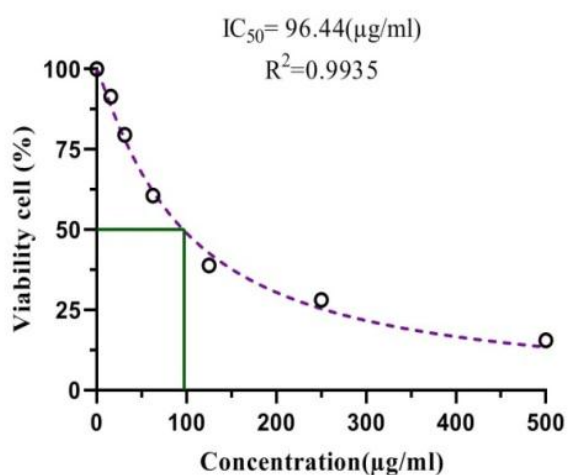


Figure 8. IC50 of free quercetin drug.

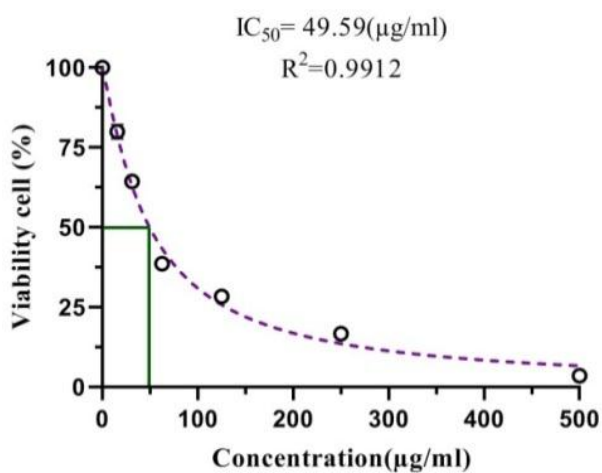


Figure 9. IC50 of Fe₃O₄ nanoparticles containing quercetin.

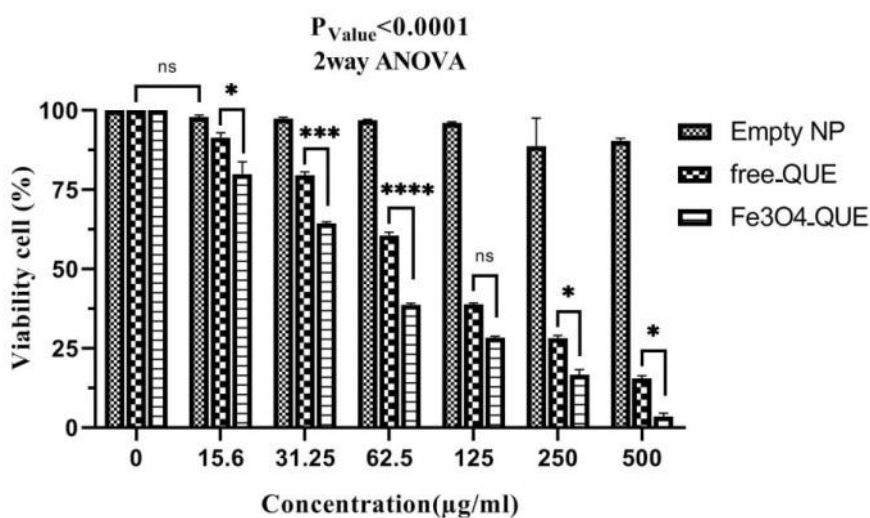


Figure 10. The percentage of MCF-7 cell survival in the presence of free quercetin.

Discussion

In addition to the fabrication of Fe₃O₄ nanosystem containing the anticancer drug quercetin, the present research investigated its physicochemical properties; the results suggested that this nanosystem had an encapsulation efficiency of 74% and a controlled release of the drug in the conditions of healthy and cancerous cells. The nanoparticle analysis with DLS device confirmed that the Fe₃O₄ nanoparticle formulation had a proper size of 91.2 nm and a zeta potential of 65.1 mV. The amount of zeta potential is an important factor in the stability of nanoparticles. With increasing the amount of zeta potential, the stability of nanoparticles increases as well; this is because enhancing surface charge enhances the repulsive force between nanoparticles and reduces the aggregation and adhesion of nanoparticles. Moreover, after investigation of the cytotoxicity of this drug-containing nanoparticle, it was found that at the same concentration, the toxicity level of encapsulated quercetin compared to free quercetin was significantly increased on the breast-cancer cell line MCF-7.

One important point of the current research is the higher rate of drug release from Fe₃O₄ nanoparticles coated with poly ethylene imine in the microenvironment of cancer cells compared to

the healthy-cells microenvironment. This difference was due to the difference in temperature and acidity between cancer cells and healthy cells, which led to more drug leakage from Fe₃O₄ coated with poly ethylene imine in cancer cell conditions; this fact can ensure that the quercetin drug is delivered to the tumor site to a greater extent.

So far, several studies have been conducted on Fe₃O₄ nanoparticles. Kievit in 2015 showed that coating the surface of Fe₃O₄ magnetic nanoparticles by PEI creates spherical morphology and increases the size of nanoparticles. The increased size of magnetic nanoparticles can make zeta potential more positive and increase the long-term colloidal stability and proper stability, enhancing the amount of drug loading by nanoparticles (Kievit and Zhang, 2011). In this study, nanoparticles coated with PEI, reached the size range of about 90-100 nm, which optimizes the amount of drug loading and zeta potential.

Coating with PEI, in addition, effectively increases nanoparticle biocompatibility, facilitates drug loading and its targeted transfer to cancerous cells, and reduces the accumulation or aggregation of nanoparticles.

In a study conducted by Jia in 2012, the DOX Fe₃O₄-PLGA-nanoparticle complex was

synthesized, showing a high loading rate; it also had a stable release with a faster release in acidic pH. These nanoparticles were applied to mouse lung cancer cells in a laboratory environment, which resulted in the apoptosis of cancer cells (Jia *et al.*, 2012). The results of this article were consistent with the results of the present study, and drug release was reported at a lower pH. In 2011, Rasouljan-broujeni prepared and evaluated nanoparticles containing oxaliplatin drug by nano deposition method. In this research, the percentage of drug loading and the size of the resulting nanoparticles have been reported 36% and 194 nm (Rasouljan-broujeni *et al.*, 2011). The difference in the type of nanoparticle, the method of making the nanoparticle, and the type of drug were among the factors that caused the difference in the results between Borujani's research and the present research. However, the fabrication of nano-systems with smaller size and higher loading percentage was one of the advantages of the current research compared to the research by Borujani. Accordingly, in this research, nanoparticles containing drugs had a much greater effect on cancer cells than the free state of the drug on MCF cancer cells.

Conclusion

This study proves that the coating of Fe₃O₄ nanosystem with PEI causes high drug absorption and storage in the nanoparticles, and also increases the stability and biocompatibility of the particles and prevents the accumulation or coagulation of the particles. Fe₃O₄ nanoparticles loaded with quercetin induce higher cell death than free quercetin alone. These nanoparticles can be used to improve the effect and reduce the toxicity of the drug after completing these tests in vivo conditions. Distribution and intracellular interactions of quercetin drug in the free state and loaded on nanoparticles may be different, and nanoparticles loaded with quercetin play an important role in causing high cytotoxicity through enhancing the production of ROS or other unknown mechanisms. In general, it can be concluded that by placing different coatings and

functional groups on Fe₃O₄ nanoparticles, they can be used in various fields such as drug delivery, killing cancer cells and other fields of medicine and biology. The present study shows the potential of Fe₃O₄ nanoparticles in designing new magnetic nanocarriers and targeting them with other molecules for drug delivery and cancer chemotherapy. A formulation was proposed for manufacturing this nanoparticle; while it has a controlled release pattern in healthy and cancerous cell conditions, its physicochemical characteristics are satisfactory; they include electrical charge, high-encapsulation capacity and a favorable size of about 90 nm, which can be suggested as a suitable carrier in anti-tumor chemotherapy.

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Authors' contributions

Sadeghi Nodoushan F and Taghiyar S were involved in practical work, statistical data analysis, writing the manuscript. Hakimian F and Haghirsadat BF supervised the research, edited the manuscript, and approved data analysis. All the authors approved the final version of the manuscript for publishing.

Conflicts of interest:

Authors declared no conflict of interest.

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